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Absence of premature senescence in Werner's syndrome keratinocytes

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ABSTRACT

Werner's syndrome (WS) is an autosomal recessive genetic disorder caused by loss of function mutation in *wrn* and is a useful model of premature *in vivo* ageing. Cellular senescence is a plausible causal mechanism of mammalian ageing and, at the cellular level, WS fibroblasts show premature senescence resulting from a combination of telomeric attrition and replication fork stalling. Over 90% of WS fibroblast cultures achieve <20 population doublings (PD) *in vitro* compared to wild type human fibroblast cultures.

It has been proposed that some cell types, capable of proliferation, will fail to show a premature senescence phenotype in response to *wrn* mutations. To test this hypothesis, human dermal keratinocytes (derived from both WS and wild type patients) were cultured long term. WS Keratinocytes showed a replicative lifespan in excess of 100 population doublings but maintained functional growth arrest mechanisms based on p16 and p53. The karyotype of the cells was superficially normal and the cultures retained markers characteristic of keratinocyte holoclones (stem cells) including p63 expression and telomerase activity. Accordingly we conclude that, in contrast to WS fibroblasts, WS keratinocytes do not demonstrate slow growth rates or features of premature senescence. These findings suggest that the epidermis is among the tissue types that do not display symptoms of premature ageing caused by loss of function of *wrn*. This is in support that Werner's syndrome is a segmental progeroid syndrome.

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1. Introduction

The ageing phenotype is a complex one, which has the potential to be moulded by a wide combination of genetic and environmental effects. Population-based studies, which seek to identify candidate genes involved in successful ageing through the study of long-lived populations, are potentially both feasible and informative but their interpretation is not always straightforward (Kirkwood, 1996; Leckie, 1992).

Alternative, and possibly simpler, approaches rely on either (i) the identification in a simple model organism of single gene mutations which significantly extend healthy lifespan (Kimura et al., 1997; Clancy et al., 2001) or (ii) the study of heritable human genetic diseases, or “progeroid syndromes”, which mimic some, but not all, features of the ageing process in order to gain insights into how the ageing process functions in normal individuals (Martin, 1982; Kipling and Faragher, 1997).

Werner syndrome (WS, MCK277700) is a well-known progeroid syndrome caused by loss of function mutation in the *wrn* gene. The enzyme is involved in DNA recombination, repair and the re-initiation of stalled DNA replication forks (Yu et al., 1996; Cox and Faragher, 2007) and the disease itself is the highest ranked candidate for a disorder which accelerates multiple features of normal human ageing (Martin, 1985; Cox and Faragher, 2007).

The challenge with using model systems to understand ageing is to separate out disease or species specific observations from those that are of general applicability to the ageing population. This requires that data, generated using models, must be evaluated within the context of theories designed to explain how normal ageing operates. In this regard, Werner syndrome is particularly utile because some tissue and organ systems (*e.g.* the cardiovascular system) are severely affected whilst in others the “ageing” pathology appears to be limited or absent (*e.g.* the immune system). Clear mechanistic hypotheses thus allow researchers to design experiments which can separate correlative relationships from those with the potential to be causal.

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Two theories, which also have explanatory power for the normal ageing process, have been used to explain the phenotypic presentation of WS patients and *wrn* knockout animals. The first places an emphasis on oxidative damage, with concomitant mutation accumulation, as the primary driver of pathology (Labbé et al., 2010, 2012). The second identifies the accumulation of senescent cells as the major causal mechanism (Ostler et al., 2002; Cox and Faragher, 2007). These theories are not mutually exclusive (Kudlow et al., 2007) but the differences in emphasis between them are large enough to give rise to different predictions in the context of Werner's syndrome. In particular, any theory used to explain the phenotype of WS must be able, at least in principle, to explain why some tissues are severely affected and others much less when the disease is caused by the loss of function of a ubiquitously expressed gene.

Senescent cells are the viable, but permanently growth arrested form of cells from mitotic populations (Burton and Faragher, 2015; Norsgaard et al., 1996). The senescent state often results from extended cell division but can also occur through cell stress or oncogene activation. A variety of molecular pathways, including critical telomere shortening, drive entry into senescence resulting in a cell with a profoundly altered phenotype. This phenotype typically involves the elevated secretion of pro-inflammatory cytokines and matrix metalloproteinases (termed the senescence associated secretory phenotype or SASP) but can also involve pro-calcificatory changes or the loss of the ability to contribute to effect immune responses (Burton and Faragher, 2015).

The cell senescence hypothesis invokes the known differences in the controls on the replicative lifespans of different cell types to explain the lack of disease phenotypes in some WS tissues. It considers senescent cells to be the primary drivers of the disease pathology and thus predicts that cell populations from affected WS tissues will show premature senescence *in vivo* whilst those derived from unaffected tissues will have a normal replicative lifespan. One of the cardinal *in vitro* features of WS is the exceptionally short replicative lifespan of dermal fibroblasts derived from patients. This is typically <20 population doublings and is the result of greatly increased rates of cell cycle exit (Faragher et al., 1993). This exit is primarily driven by intra-S phase arrest. However, abbreviated replicative lifespans are not universal among WS cell populations. This has been demonstrated in cultures of T cells derived from WS patients, which did not show a difference in replicative lifespans compared to normal controls (James et al., 2000).

The normal proliferative capacity of WS T cells may be attributed to the natural expression of telomerase in this cell type (Bodnar et al., 1996). Several studies have shown that telomerase expression can have a protective effect that prevents premature senescence in WS. For instance, ectopic expression of telomerase results in lifespan extension of WS fibroblasts (Wyllie et al., 2000). Reprogramming WS cells to specific lineages that naturally express telomerase also result in lifespan extension as was demonstrated in induced pluripotent and neuronal progenitor cells (Shimamoto et al., 2014; Cheung et al., 2014).

In keratinocytes, senescence does not take place directly, the cells must undergo clonal evolution from stem cell to transient amplifying cells prior to growth arrest. Keratinocytes in the basal layer naturally express telomerase and are known to grow in stem cell colonies (holoclones). Keratinocyte stem cells then stochastically divide into stem cells or transient amplifying cells. The transient amplifying cell population can undergo a series of divisions before they either senesce or commit to terminal differentiation (Watt, 1998; Barrandon and Green, 1987; Dellambra et al., 2000).

In Werner's syndrome, dermal fibroblasts enter premature senescence and features of dermal skin disorders mainly scleroderma-like skin, are evident. In contrast to the dermis, the epidermal layer of the skin appears to be less effected in WS patients (Epstein et al., 1966; Goto, 1997). Accordingly the cell senescence hypothesis was tested by culturing and characterising the growth and phenotype of keratinocytes derived from the epidermal basal layer of WS patients. WS keratinocytes were grown in parallel to wild type keratinocytes under optimum conditions (co-cultured with feeder cells) to promote proliferation and maintenance in the stem cell compartment (Rheinwald and Green, 1977). WS cells were then examined for normal keratinocyte characteristics including differentiation, senescence and stem cell properties of the basal layer. This study is aimed to improve our understanding of the state of keratinocytes in WS and its involvement in the premature age pathology of WS.

2. Materials & methods

2.1. Initiation and culture of primary human keratinocytes

Primary keratinocytes were isolated mainly from the basal layer of the epidermis by Drs M. Illsley and S.E. James at the Blonde McIndoe Centre, East Grinstead, UK (James et al., 2010). The strains were obtained from 26 year old male patient (SK206AK) and 26 year old female Werner's syndrome patient (WSK368).

Keratinocytes were routinely seeded at densities between 1.2×10^4 and 2×10^4 cells/cm^2 co-cultured with a layer of γ-irradiated (9000 rads) 3T3 cells (mouse embryonic fibroblasts, National Institute for Biological Standards and Control, Hertfordshire, UK) seeded at $2.4 \times 10^4 - 4 \times 10^4$ cells/cm^2. The cells were cultured in Rheinwald and Green culture medium (60% v/v Dulbecco's modified Eagle's medium (DMEM: Gibco®, Life Technologies, Paisley, UK), 20% (v/v) Ham's F12 (Gibco®, Life Technologies) and 20% (v/v) Foetal Bovine Serum (Gibco®, Life Technologies) supplemented with 10 ng/ml epidermal growth factor (EGF) (Sigma-Aldrich Company Ltd., Poole, UK), 400 ng/ml hydrocortisone (Sigma-Aldrich Company Ltd.), and 10^{-10} M cholera toxin (Sigma-Aldrich Company Ltd.), (Green et al., 1977; Rheinwald and Green, 1977). The medium was replaced every 2 to 3 days and the keratinocytes were passaged when they had reached 80% confluency. Keratinocytes were co cultured with feeder cells in all experiments, except when transferred to defined keratinocyte serum free media (K-SFM) (Gibco®, Life Technologies) to test the reactions of cells to change in culture conditions. Cells were maintained at 37 °C in a humidified atmosphere containing 10% CO_2 and 90% air. The number of population doublings (PD) per passage were calculated and plotted against the total time in culture ($PD = \log(N_t / N_o) / \log 2$, where N_t is number of cells counted and N_o is number of cells seeded).

For growth arrest studies, keratinocytes were treated with 1 μM Adriamycin (doxorubicin) ($C_{27}H_{29}NO_{11}$) (Sigma-Aldrich Company Ltd.) for 2 h followed by replacement with drug free media.

2.2. Senescence-associated β-galactosidase assay (SA-β-gal)

Keratinocytes were grown in a 12 well plate (Iwaki®, Sterilin Ltd., Cambridge, UK) for 48 h and fixed with 3% formalin (Sigma-Aldrich Company Ltd.). Senescence-associated β-galactosidase staining was carried out as described by Dimri and co-workers (1995).

2.3. Indirect immunofluorescence

Keratinocytes were seeded on 13 mm coverslips (VWR International, Radnor, PA, USA) and maintained to approximately 60–80% confluence. The samples were fixed with a 1:1 methanol:acetone solution, except for WRN detection, where the cells were fixed with 4% paraformaldehyde followed by permeablization in 0.1% Triton X-100. The cells were then incubated in primary antibody solutions overnight at 4 °C and in secondary antibody solutions for 45 min at room temperature. For Ki67 labelling polyclonal rabbit anti-human Ki67 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA); 1:30 followed by FITC conjugated swine anti-rabbit IgG (Dako Agilent Technologies, Glostrup, Denmark); 1:30 were used. For cytokeratin staining monoclonal mouse anti-human cytokeratin (Dako Agilent Technologies); 1:30 followed by FITC conjugated rabbit anti-mouse IgG (Dako Agilent Technologies); 1:30 were used. For involucrin labelling monoclonal mouse anti-

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